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Postshock Mesenteric Lymph Induces Endothelial NF-κB Activation

Sagar S. Damle, M.D.,* Ernest E. Moore, M.D.,†,[1] Trevor L. Nydam, M.D.,* Monesha Banerjee,* Fabia Gamboni-Robertson, Ph.D.,* Xin Su, M.D.,* and Anirban Banerjee, Ph.D.*

**University of Colorado at Denver, Health Sciences Center, Denver, Colorado; and †Denver Health Medical Center, Denver, Colorado*

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Background. **Posthemorrhagic shock mesenteric lymph (PSML) has been shown to activate pulmonary endothelial cells and cause lung injury. Although multiple mediators may be involved, most of these effects are mediated by nuclear factor-kappa B (NF-κB) activation. Degradation of the inhibitor of kappa B (IκB) is a key regulatory step in the activation of NF-κB. We therefore hypothesized that PSML would cause IκB degradation with subsequent NF-κB phosphorylation and nuclear translocation.**

Methods. **Mesenteric lymph was collected from male rats before shock and each hour after shock for up to 3 h (n = 5). Buffer (control), buffer + 10% (v/v) lymph, or buffer + tumor necrosis factor (10 ng/mL) were incubated with human pulmonary endothelial cells for 30 min and then lysed. Immunoblots of lysates were probed for IκB and phospho-p65. Immunohistochemistry was performed on cells grown on glass slides and then treated as above with the third PSML sample. Cells were fixed and then probed for p65. Statistical analysis was performed with Student's t-test and analysis of variance with significance was set at $P < 0.05$.**

Results. **Western blots of cell lysates for IκB demonstrated a steady decrease in total IκB with each lymph sample. Phosphorylation of NF-κB , p65 component, steadily increased with each PSML sample, with a maximum reached during the third PSML sample, which also significantly increased translocation of NF-κB to the nucleus.**

Conclusion. **Postshock mesenteric lymph bioactivity is mediated by pathways which involved IκB degradation. These pathways offer novel off targets for clinical intervention to prevent the distal organ injury caused by postinjury hemorrhagic shock.**

Key Words: **mesenteric; lymph; shock; IκB, NF-κB.**

INTRODUCTION

Gut ischemia and reperfusion (I/R) is central to the pathogenesis of multiple organ failure after hemorrhagic shock. Although early work focused on the portal circulation as the conduit for biological mediators, more recently, mesenteric lymph has been identified as the mechanistic link between gut I/R and neutrophil-mediated acute lung injury [1–3].

Both *in vivo* and *in vitro* work have focused on the role of neutrophils (polymorph nuclear leukocytes [PMNs]) in mediating lung injury after hemorrhagic shock. We have shown that in a rodent model of hemorrhagic shock, PMN adhesion molecule expression and PMN lung accumulation are diminished with lymph diversion [4]. Further corroborative work *in vitro* has shown that isolated PMNs exposed to postshock mesenteric lymph (PSML) increase surface adhesion molecule expression and increase priming for superoxide production [5].

Although PMNs play a key role in lung injury [6], activation of the pulmonary vascular endothelium is an essential component [6]. The Deitech lab has found that human umbilical vein endothelium (HUVECs) exposed to PSML undergoes cytotoxicitiy and increased permeability [2, 7]. We have shown that pulmonary microvascular endothelial cells (HMVECs) profoundly up-regulate surface expression of intercellular adhesion molecule-1 (ICAM-1) after exposure to PSML [8].

This increase in ICAM-1 has prompted us to examine the role of nuclear factor-kappa B (NF-κB)-mediated pathways, since NF-κB is a key regulator of the ICAM-1 gene as well as several other pro-inflammatory genes [9].

[1] To whom correspondence and reprint requests should be addressed at Denver Health Medical Center/University of Colorado Health Sciences Center, Department of Surgery, 777 Bannock Street, Denver, CO 80204. E-mail: Ernest.Moore@dhha.org.

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Components of the active NF-κB are sequestered in the cytosol by specific proteins of the inhibitor of kappa B (IκB) family [10, 11]. Upon phosphorlyation of IκB, NF-κB components are released and IκB is degraded [12]. Liberation of the NF-κB components allows the molecules to translocate to the nucleus, where they dimerize and initiate transcription of key genes, including ICAM-1 [9, 12]. Recently, phosphorylation of NF-κB, specifically phosphorylation at the serine 536 site of p65 RelA, by I kappa B kinase (IκK) has emerged as a novel mechanism of transcriptional activation [13].

We therefore hypothesized that endothelial activation by PSML would involve degradation of IκB with subsequent phosphorylation and translocation of NF-κB to the nucleus in HMVECs.

METHODS

Animals

Animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee at Denver Health Medical Center, Denver, Colorado. Adult male Sprague Dawley rats (Harlan Laboratories, Madison, WI) weighing 320–370 g were housed under barrier-sustained conditions and allowed free access to chow and water before use. All animals were maintained in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals*.

Materials

All materials were purchased from Sigma-Aldrich Corp. (St. Louis, MO), unless otherwise specified. All tubing was obtained from Fisher Scientific (Pittsburgh, PA). Pentobarbital sodium was purchased from Abbott Labs (North Chicago, IL) and heparin from American Pharmaceutical Partner Inc. (Schaumburg, IL). Antibodies against IκBα (C-21) and p65 (C-20) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against actin (AC-40) were purchased from Abcam, Inc. (Cambridge, MA). Antibodies against phosph-S536-p65 (93H1) were obtained from Cell Signaling Technology, Inc. (Danvers, MA).

Hemorrhagic Shock Model

Rats were anesthetized with pentobarbital 50 mg/kg intraperitoneally. The right femoral artery and vein were cannulated with PE-50 tubing. Blood pressure was continuously monitored with a Pro-Paq device (Protocol Systems, Beaverton, OR). Core body temperature was measured with a rectal thermometer and maintained at 37°C with a heating lamp. A midline laparotomy was performed to expose the common mesenteric lymphatic duct, which was cannulated with 0.02-in silicone tubing. The tubing was secured with a 7-O Prolene suture and exteriorized via a right flank stab wound. The abdomen and groin were then closed with 4-O monofilament nylons.

Lymph was collected from rats for 60 min before (preshock), during nonlethal hemorrhagic shock (mean arterial pressure 30 mm Hg × 40 min), and for each hour after shock up to 3 h (1st hour PSML, 2nd hour PSML, 3rd hour PSML). Animals were resuscitated during the initial 2 h after shock with half-shed blood volume +4× shed blood volume as normal saline. Lymph was centrifuged at 6,000 *g* × 10 min at 4°C to remove all cellular components. The supernatant was removed, aliquoted, and snap frozen for subsequent analysis.

Cell Culture

HMVECs were obtained from Cambrex Bio Science Inc. (Rockland, ME) and cultured in endothelial growth medium. Cells were grown to near confluence at 37°C in 5% CO_2. After achieving near confluence, cells were incubated with medium, tumor necrosis factor (TNF) (10 ng/mL), or lymph (10% v/v) for 30 min at 37°C. To minimize animal-to-animal variability, all postshock mesenteric lymph samples were compared to the same animal's preshock sample. Supernatants were removed, and cells were lysed in digestion buffer containing SDS. Cell lysates were heated at 100°C for 10 min, aliquoted, and frozen for immunoblot analysis.

Immunoblot Staining

Equal amounts of whole cell lysates were loaded into 8–16% acrylamide gels (Pierce Biotechnology, Inc., Rockford, IL), fractioned with SDS-PAGE, and then electrotransferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Rockford, IL). Membranes were blocked with 5% nonfat milk solution and immunoprobed. Bound antibodies were detected with enhanced chemoluminescence detection system (Pierce Biotechnology, Inc.). Densitometry was performed using Image J software program (National Institutes of Health, Bethesda, MD). All plots were normalized to actin and results expressed as a percent of control.

Immunofluorescent Microscopy

HMVECs were grown on Lab-Tek Chamber Slides (Nalgene Nunc International, Rochester, NY) and treated with buffer, TNF (ng/mL), or 3rd hour PSML (10% v/v) for 45 min. The slides were fixed and permeabilized with 70% acetone/30% methanol solution at −20°C. Nonspecific antigen sites were blocked with 10% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in phosphate-buffered saline (PBS) for 60 min at room temperature. Slides were then probed with rabbit polyclonal anti-p65 antibody (C-20, Santa Cruz Biotechnology, Inc.) in 1% bovine serum albumin/PBS for 1 h. Negative control slides were incubated with isotype (Jackson ImmunoResearch Laboratories). Following three PBS washes, the slides were incubated for 1 h at room temperature with donkey anti-rabbit Cy3-conjugated antibody (Jackson ImmunoResearch Laboratories) and bis-benzimide nuclear stain (Sigma-Aldrich).

Images were acquired with a Zeiss Axiovert fitted with a Cooke CCD SensiCam using a Chroma Sedat with single excitation and emission filters and a multiple bandpass dichroic and Sutter filter control. The two channel images were then digitally processed using Intelligent Imaging Innovations Slidebook 4.1 software (Intelligent Imaging Innovations, Inc., Denver, CO). Nuclear stains were masked to measure the relative p65 mean fluorescence intensity (MFI) within the nuclei. The MFI of at least 70 cells from each experimental group were analyzed by analysis of variance (ANOVA) using JMP 5.0.1 software. Statistical significance was accepted as P less than 0.001.

Statistical Analysis

Data were analyzed by repeated measures ANOVA using Proc GLM, followed by the Tukey's multiple comparisons test (SAS Tutorials for Statistical Data Analysis at http://www.stattutorials.com/SAS/). Contrasts were used to test for linear trends between hours pre/postshock and IκB degradation as well as hours pre/postshock and the phosphorylation state of the p65 component. A P value of less than 0.05 is considered statistically significant.